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DEPARTMENT OF THE ARMY
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# INACTIVATION OF PURIFIED VENEZUELAN EQUINE ENCEPHALITIS VIRUS BY IONIZING RADIATION

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Medical Investigation Division
MEDICAL SCIENCES LABORATORIES

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In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

## ACKNOWLEDGMENT

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## ABSTRACT

Purified virus preparations with increased specific antigen concentration and minimal nonantigenic constituents are favored for the development of virus vaccines. Venezuelan equine encephalitis (VEE) virus was purified and concentrated by chromatography of tissue culture supernatant fluids on diethylaminoethyl (DEAE) cellulose columns. Initial stepwise gradient elution studies indicated a broad elution pattern for the virus, with recovery from 0.05 to 0.70 M NaCl. Optical density, infectivity, hemagglutination (HA), and complement fixation (CF) assays indicated that complete recovery of input virus in highly purified form was possible. Single-step elution with 0.7 M tris-succinate-salt buffer resulted in a virus volume decrease of 85% with a concomitant increase in infectivity and antigenicity. Recoveries consistently equaled or exceeded 100% of the input preparations. Additional purification of column-recovered virus was obtained by sedimentation of pooled virus eluates on 50% sucrose cushions. Exposure of borate saline and 0.5% histidine suspensions of purified VEE virus preparations to $6 \times 10^6$ r gamma radiation resulted in loss of infectivity for tissue culture and loss of lethality for weanling and suckling mice. Inactivation was an exponential function of the dosage. In contrast, antigenicity (HA and CF) of both saline and histidine preparations was retained after irradiation with doses up to $6 \times 10^6$ r. Purified and irradiated VEE virus preparations have been used successfully for routine serological tests and are being evaluated as vaccines.

## I. INTRODUCTION*

Numerous studies have demonstrated that a variety of microbial agents can be killed by ionizing radiation with greater retention of antigenicity than than similar preparations killed by chemicals or heat.[1-4] This method of inactivation has also been shown to destroy the infectivity of Venezuelan equine encephalitis (VEE) virus - containing tissue culture supernatant fluids while retaining the virus' capacity to stimulate antibody production.[5] Purified virus preparations with increased specific antigen concentration and minimal nonimmunogenic constituents are favored for the development of virus vaccines. Recent studies indicated that arbovirus preparations of high purity and concentration were obtainable by chromatography on diethylaminoethyl (DEAE) cellulose columns.[6,7] These factors prompted this investigation of inactivation of column-purified VEE virus by ionizing radiation.

## II. MATERIALS AND METHODS

### A. VIRUS

The virus used in this investigation was the Trinidad strain of VEE virus, originally isolated in guinea pigs from a donkey brain. It had been passed 13 times in chicken embryos when it was obtained through the courtesy of William P. Allen. Working seeds for these studies were prepared after two mouse brain passes as 10% suspensions of infected suckling mouse brains in borate saline.

### B. INFECTIVITY AND ANTIGENICITY DETERMINATIONS

Assays for virus infectivity were performed by plaque titration in 24-hour chick embryo monolayer tissue cultures. Samples of irradiated virus that failed to produce plaques and were nonlethal for weanling mice were titrated in suckling mice by intracerebral inoculation of 0.02 ml. Assays for virus antigenicity were performed by hemagglutination and complement fixation tests using microtiter methods.[8,9] Hemagglutination was tested with goose erythrocytes at pH 5.8; the National Communicable Disease Center Laboratory Branch Complement Fixation (LBCF) procedure was employed in complement fixation tests.[10]

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## C. TISSUE CULTURE VIRUS

Starting virus preparations for column purification studies were from infected tissue culture supernatant fluids of 24-hour chick embryo monolayer cultures. The multiplicity of infection was approximately 0.1 to insure several cycles of virus growth in the tissue culture. The cultures were harvested by aspiration at 18 to 20 hours postinfection, and tissue culture cellular debris was removed by slow-speed centrifugation. Prior to adding the clarified virus preparations to the columns they were dialyzed for 18 hours at 5 C against 10 volumes of water to decrease their salt concentration. This procedure did not materially decrease VEE plaque titer or antigenicity.

## D. DEAE CELLULOSE COLUMN CHROMATOGRAPHY

Columns for chromatography were prepared of 10 g DEAE cellulose. Prior to use they were washed and equilibrated with 0.01 M NaCl in 0.01 M phosphate buffer, pH 7.2. Column dimension ratios, length to diameter, were approximately 2:1. Adsorption of the virus preparation was by free gravity flow, but during development of the column the flow rate was maintained between 5 and 10 ml per minute.

## E. IRRADIATION

Purified virus preparations were irradiated at the National Bureau of Standards through the courtesy of Daniel W. Brown. Suspensions of VEE virus were exposed to gamma radiation from a cobalt-60 source emitting radiation at a dose rate of $7 \times 10^4$ r per minute. The suspensions were irradiated in 4-ml quantities in 12 x 75 mm polypropylene test tubes. Samples were kept frozen with dry ice during irradiation and were held in the frozen state until tested for infectivity. The different doses of radiation were obtained by varying the time of exposure at the constant dose rate. Control samples of virus were exposed to identical conditions but were not irradiated.

## III. RESULTS AND DISCUSSION

### A. ELUTION BY THE STEPWISE METHOD

Investigation of fractionation of VEE virus was initiated by a study of its elution pattern during a stepwise procedure. After the starting tissue culture - virus effluent was collected, the virus was eluted with NaCl solutions of increasing concentrations. Fractions of eluates (250 ml each) from each of the various concentrations of NaCl solution were collected. Optical density, infectivity, and hemagglutinating activity of each fraction were determined. Results are given in Table 1. Approximately 90% of input virus infectivity and all hemagglutinating activity were retained by the column before elution was initiated. In contrast, approximately 80% of the light-absorbing components of the preparation were recovered in the effluent. Virus infectivity and antigenicity were not recovered with 0.01 M NaCl elution, while additional light-absorbing components were again eluted. VEE virus was eluted in a range of salt concentrations from 0.05 M to 0.70 M. The eluates obtained from this range showed different levels of virus content, with the greatest elution at 0.05, 0.10, and 0.5 M salt concentrations. Table 2 shows the percentage recovery of virus infectivity. These data suggest a variation in the degree of binding to DEAE in the virus population. Essentially all infectivity was recovered by elution up to 0.70 M salt. As a result of these findings, it was considered probable that a virus sample of relatively high purity and concentration might be obtained by removing contaminating tissue culture material with a salt solution of low molarity and then collecting the virus in one step with a developing buffer of relatively high concentration.

### B. CHROMATOGRAPHIC PURIFICATION OF VEE VIRUS

The typical recovery of purified VEE virus by such methodology is shown in Table 3. After addition of virus, the columns were washed with 0.01 M NaCl in 0.01 M phosphate buffer, pH 7.2. Then the virus was eluted with a 0.7 M tris-succinate-salt buffer at pH 10. Usually, 25-ml fractions were collected. Optical density, infectivity, HA and CF activity of the collected samples were determined. Infectious virus was recovered immediately after the column void volume. These fractions were characterized by high light-scattering capability. Chromatography resulted in a virus volume decrease from 500 ml to 75 ml (85%) with a concomitant increase in plaque-forming infectivity to represent essentially complete recovery of the total input virus preparation. Excellent correlation was found among the various tested properties. Those fractions with high infectivity also had the greatest HA and CF activity. Representative column recoveries of infectivity and serological activity are indicated in Table 4. Recovered infectivity and antigenicity consistently equaled or exceeded 100% of the total input preparations. The mean recovery of infectivity was 115%. The increased infectivity may represent a virus with greater efficiency for the tissue culture, or may reflect the removal of some infectivity-inhibiting substance.

TABLE 1. STEPWISE ELUTION OF VEE VIRUS FROM DEAE CELLULOSE

| Material | Volume, ml | $OD_{280}$[a] | Infectivity[b] | HA[c] |
|---|---|---|---|---|
| Virus preparation | 500 | >2.00 | 8.2 | 256 |
| Tissue culture - virus effluent | 500 | >2.00 | 6.3 | 0 |
| 0.01 M NaCl | 250 | 2.00 | 6.6 | 0 |
| 0.01 M NaCl | 250 | 0.38 | 6.0 | 0 |
| 0.05 M NaCl | 250 | 0.73 | 8.0 | 256 |
| 0.05 M NaCl | 250 | 0.31 | 6.9 | 8 |
| 0.10 M NaCl | 250 | 0.70 | 8.2 | 64 |
| 0.10 M NaCl | 250 | 0.27 | 6.5 | 4 |
| 0.20 M NaCl | 250 | 0.60 | 7.3 | 8 |
| 0.20 M NaCl | 250 | 0.22 | 6.5 | 2 |
| 0.50 M NaCl | 250 | 0.46 | 7.7 | 32 |
| 0.50 M NaCl | 250 | 0.09 | 6.5 | 2 |
| 0.70 M NaCl | 250 | 0.05 | 6.7 | 0 |
| 0.70 M NaCl | 250 | 0.00 | 6.5 | 0 |

a. Optical density at 280 mμ.
b. $Log_{10}$ pfu/ml on 24-hour chick embryo monolayers.
c. Agglutination of goose erythrocytes at pH 5.8; 0.05 ml.

TABLE 2. VARIATION OF BINDING TO DEAE OF VEE VIRUS[a]

| NaCl Elution | Infectivity[b] | Input Recovered, % |
|---|---|---|
| 0.01 M | 6.6 | 1.3 |
| 0.05 M | 8.0 | 33.3 |
| 0.10 M | 8.2 | 49.3 |
| 0.20 M | 7.3 | 5.3 |
| 0.50 M | 7.7 | 14.6 |

a. Input virus infectivity $log_{10}$ pfu/ml 8.2.
b. $Log_{10}$ pfu/ml.

TABLE 3. RECOVERY OF PARTIALLY PURIFIED VEE VIRUS BY DEAE CELLULOSE CHROMATOGRAPHY

| Material | Volume, ml | $OD_{280}$[a] | Infectivity[b] | HA[c] | CF[d] |
|---|---|---|---|---|---|
| Virus sample | 500 | 0.63 | 8.2 | 256 | 16 |
| Virus effluent | 500 | 0.57 | 5.5 | 0 | 0 |
| NaCl wash (1) | 500 | 0.16 | 4.0 | 0 | 0 |
| NaCl wash (2) | 500 | 0.00 | <4.0 | 0 | 0 |
| Fraction 1 | 25 | 0.00 | <4.0 | 0 | 0 |
| 2 | 25 | 0.00 | <4.0 | 0 | 0 |
| 3 | 25 | 0.00 | 5.9 | 4 | 0 |
| 4 | 25 | 1.65 | 9.2 | 4,096 | 256 |
| 5 | 25 | 1.25 | 9.1 | 4,096 | 256 |
| 6 | 25 | 0.39 | 8.8 | 1,024 | 32 |
| 7 | 25 | 0.00 | 7.3 | 64 | 8 |
| 8 | 25 | 0.00 | 6.6 | 8 | 4 |

a. Samples diluted 1:10.
b. $Log_{10}$ pfu/ml on 24-hour chick embryo monolayers.
c. Agglutination of goose erythrocytes at pH 5.8; 0.05 ml.
d. LBCF test with 0.025 ml.

TABLE 4. DEAE COLUMN YIELD OF INPUT VEE VIRUS INFECTIVITY AND SEROLOGICAL ACTIVITY

| | Input Recovered, % | | |
|---|---|---|---|
| Column | pfu[a] | HA[b] | CF[c] |
| A | 100 | 180 | 170 |
| B | 140 | 180 | 112 |
| C | 106 | 116 | 94 |

a. Plaque-forming units.
b. Agglutination of goose erythrocytes.
c. Complement fixation.

## C. SEDIMENTATION ON SUCROSE CUSHIONS

Column-recovered virus was additionally purified by sedimentation of pooled virus eluates on 50% sucrose cushions. Sedimentation was obtained by centrifugation at 27,000 rpm for 2.5 hours. The thin light-scattering bands recovered from above the sucrose cushions represent highly purified virus material. Usually, the final virus preparation was 1/50th the volume of the initial tissue culture material. Typical recoveries are indicated in Table 5. Although not all of the pooled material from the columns was recovered on the sucrose bands, the additional purification obtained justified the procedure. It should be noted here that 77% mean recovery of the column peaks represents essentially 90% of the total input virus in highly purified form.

TABLE 5. RECOVERY OF VEE VIRUS BY CENTRIFUGATION ON A 50% SUCROSE CUSHION

| Experiment | Input Recovered, % | | |
|---|---|---|---|
| | Infectivity[a] | HA[b] | CF[c] |
| A | 52 | 20 | 40 |
| B | 119 | 160 | 133 |
| C | 60 | 107 | 58 |
| Mean | 77 | 96 | 77 |

a. Plaque-forming units.
b. Agglutination of goose erythrocytes at pH 5.8.
c. LBCF test.

## D. IRRADIATION

The loss of infectivity and antigenicity of purified VEE virus as a function of dose of radiation was studied in borate saline and in 0.5% histidine suspensions. Suspensions were irradiated at a constant dose rate for such periods that exposures ranged from 500,000 to 6,000,000 r. Results of infectivity assays in tissue culture are presented in Figure 1. Inactivation of purified VEE virus was linear, indicating first-order behavior of the reaction. Similar linearity of inactivation was previously reported for VEE virus[8] and for influenza virus.[11] Survival of VEE virus infectivity was enhanced in the presence of histidine. This is consistent with previous findings with other viruses.[11] Both saline and histidine preparations lacked plaque-forming capacity after irradiation at 4,000,000 and 6,000,000 r. However, Figure 1 indicates that virus inactivation in histidine was not complete at 4,000,000 r. Virus inactivation was also tested in weanling mice. Results obtained, and a comparison with the plaque assay, are presented in Table 6. Deaths did not occur in weanling mice inoculated with virus exposed to 4,000,000 and 6,000,000 r.

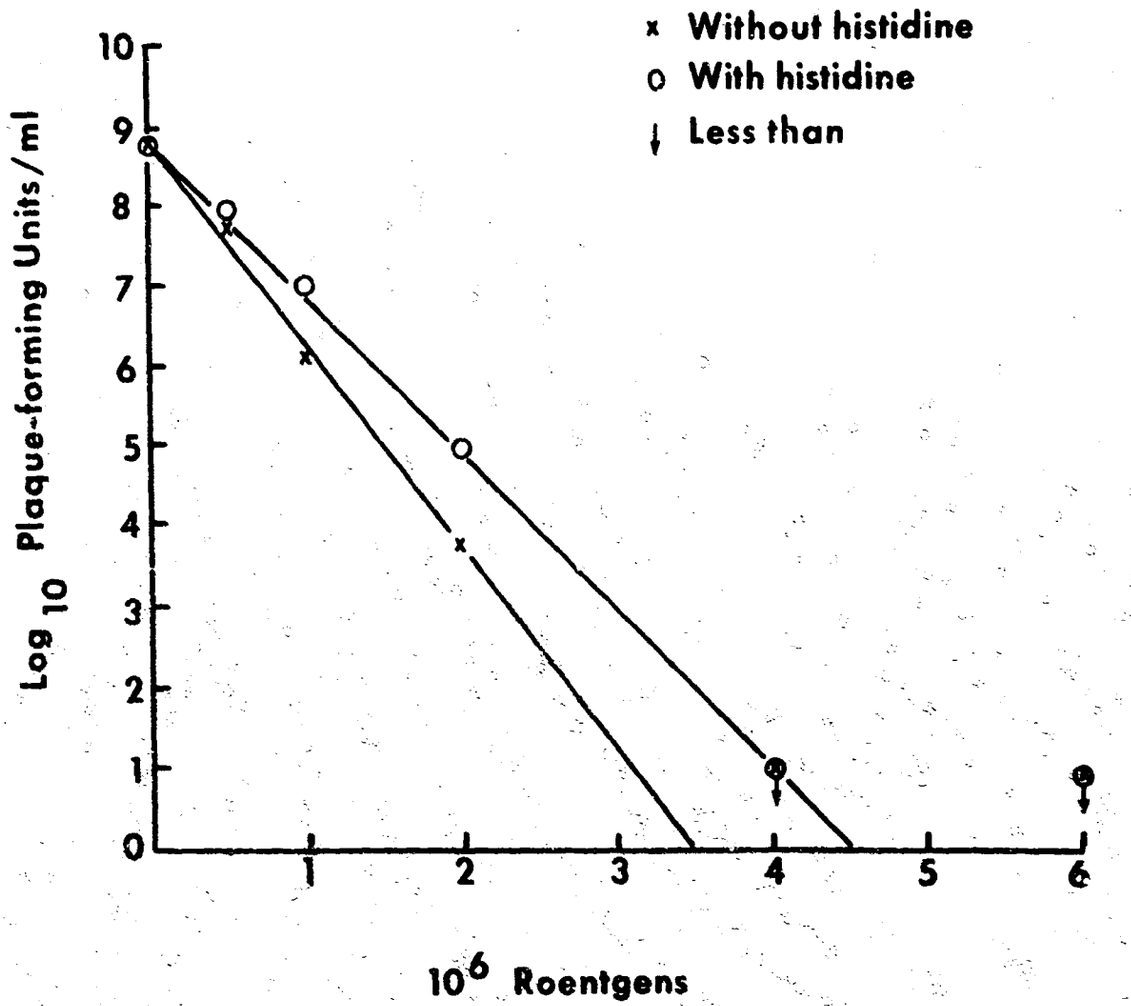


FIGURE 1. Inactivation of Purified Venezuelan Equine Encephalitis Virus by Gamma Radiation.

Inactivation curves had slopes virtually identical to those obtained with the tissue culture assay and indicated potentially viable virus in both of the 4,000,000-r preparations. Intracerebral inoculation of suckling mice confirmed the presence of infectious virus in both saline and histidine preparations irradiated at 4,000,000 r. In contrast, deaths did not occur in newborn mice inoculated with undiluted saline or histidine preparations exposed to 6,000,000 r.

TABLE 6. INACTIVATION OF PURIFIED VENEZUELAN EQUINE ENCEPHALITIS VIRUS BY IONIZING RADIATION

| $10^6$ Roentgens | Saline Preparation | | Histidine Preparation | |
|---|---|---|---|---|
| | $Log_{10}$ pfu/ml | $Log_{10}$ $MICLD_{50}$/ml | $Log_{10}$ pfu/ml | $Log_{10}$ $MICLD_{50}$/ml |
| 0 | 8.9 | 10.5 | 8.9 | 10.5 |
| 0.5 | 7.7 | 9.1 | 7.95 | 9.2 |
| 1.0 | 6.1 | 8.0 | 7.0 | 8.2 |
| 2.0 | 3.8 | 5.3 | 4.95 | 6.5 |
| 4.0 | <1.0 | <1.5 | <1.0 | <1.5 |
| 6.0 | <1.0 | <1.5 | <1.0 | <1.5 |

Previous work demonstrated that the hemagglutinin of purified influenza virus suspensions was inactivated by gamma radiation more rapidly than infectivity.[11] Addition of ring structure compounds increased the stability of both the hemagglutinin and infectivity. Histidine provided selectively greater protection to the antigen during inactivation by gamma radiation. Results of antigenicity assays of saline and histidine suspensions of purified VEE virus are presented in Figure 2. In contrast to infectivity, the antigenicity of both saline and histidine preparations of purified VEE virus were retained after irradiation at doses up to 6,000,000 r. Histidine suspensions of purified VEE virus completely retained their capacity to produce hemagglutination and complement fixation. At 6,000,000 r, the dose at which all assay systems indicated complete loss of infectivity, titers were identical to those of nonirradiated control preparations. Saline suspensions were similarly active. Although frequently (as indicated in Figure 2) an initial decrease in titer occurred after exposure to low doses of gamma radiation, inactivation did not continue linearly, and a high level of antigenicity was maintained throughout exposure to the higher doses. In these instances, at 6,000,000 r, 50% of the hemagglutinating and complement-fixing properties were still retained. More commonly, the saline-virus preparations behaved like those of histidine, with no decrease in titer at doses up to 6,000,000 r.

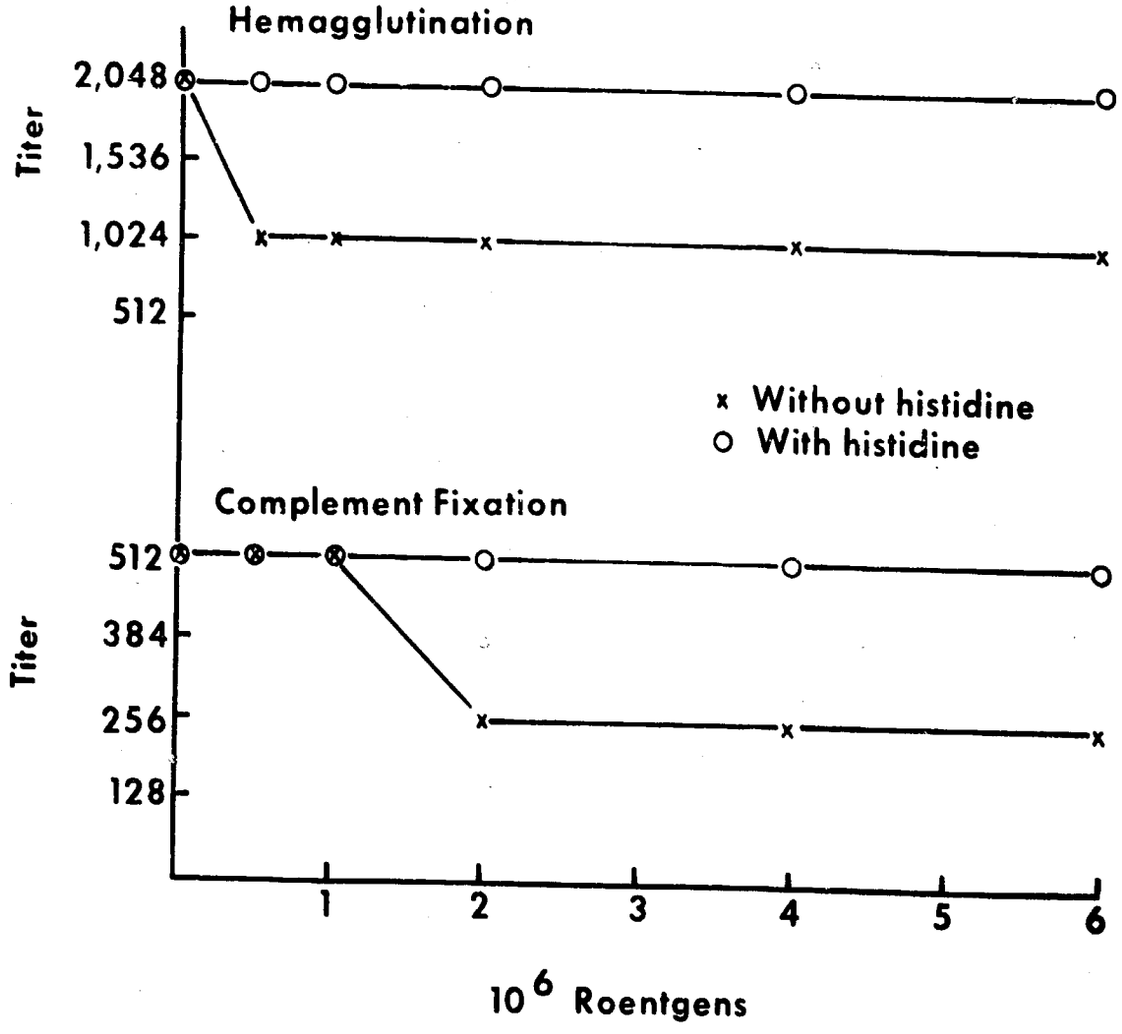


FIGURE 2. Retention of Antigenicity of Purified Venezuelan Equine Encephalitis Virus Preparations Rendered Noninfectious by Gamma Radiation.

Irradiated VEE antigens exposed to 6,000,000 r have been employed in our laboratory in both hemagglutination-inhibition and complement fixation tests of animal sera. Results were similar to those obtained with live virus preparations and with saline extracts of infected suckling mouse brains inactivated with 0.3% beta-propiolactone. No change in HA or CF titer was noted with column-purified antigens stored for 3 weeks at 5 C, and only a fourfold decrease in titer was noted after 10 weeks. Studies are currently in progress to evaluate the immunogenicity of these purified VEE preparations.

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13. ABSTRACT

Purified virus preparations with increased specific antigen concentration and minimal nonantigenic constituents are favored for the development of virus vaccines. Venezuelan equine encephalitis (VEE) virus was purified and concentrated by chromatography of tissue culture supernatant fluids on diethylaminoethyl (DEAE) cellulose columns. Initial stepwise gradient elution studies indicated a broad elution pattern for the virus, with recovery from 0.05 to 0.70 M NaCl. Optical density, infectivity, hemagglutination (HA), and complement fixation (CF) assays indicated that complete recovery of input virus in highly purified form was possible. Single-step elution with 0.7 M tris-succinate-salt buffer resulted in a virus volume decrease of 85% with a concomitant increase in infectivity and antigenicity. Recoveries consistently equaled or exceeded 100% of the input preparations. Additional purification of column-recovered virus was obtained by sedimentation of pooled virus eluates on 50% sucrose cushions. Exposure of borate saline and 0.5% histidine suspensions of purified VEE virus preparations to $6 \times 10^6$ r gamma radiation resulted in loss of infectivity for tissue culture and loss of lethality for weanling and suckling mice. Inactivation was an exponential function of the dosage. In contrast, antigenicity (HA and CF) of both saline and histidine preparations were retained after irradiation with doses up to $6 \times 10^6$ r. Purified and irradiated VEE virus preparations have been used successfully for routine serological tests and are being evaluated as vaccines.

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| Purification | | | | | | |
| Chromatography, DEAE-cellulose | | | | | | |
| Infectivity | | | | | | |
| Antigenicity | | | | | | |
| Ionizing radiation | | | | | | |
| Irradiation | | | | | | |
| Inactivation | | | | | | |
| Histidine | | | | | | |

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